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IMMUNOASSAY USING ANTIGEN–ENZYME CONJUGATES

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1. Introduction

The development of the radio-immunoassay, initiated by Berson et al. [1], has permitted estimation of antibodies and antigens to a high degree of sensitivity. This is particularly true in endocrinology, where bioassays are often difficult, time consuming and expensive.

In this type of immunoassay, the antigen occupies a central position [2] but its label, need not be a radionuclide. Haimovich et al. used protein–bacteriophage conjugates for the assay of antibodies and antigens [3, 4] and the preparation of enzyme–antibody and enzyme–antigen conjugates, mainly for histological purposes has been described [5, 6].

We have conjugated the antigen human chorionic gonadotrophin (HCG) to the enzyme horse radish peroxidase (HRP) and used purified conjugates for 'enzyme-immunoassay' of antibody and antigen**.

2. Materials and methods

2.1. *Reagents*

HCG was prepared by van Hell et al., according to procedures previously described [7, 8]. Antisera were raised in rabbits against highly purified HCG as described earlier [9]. Antiserum against rabbit γ-globin was prepared by immunization of a sheep with a γ-globulin preparation obtained by DEAE-cellulose chromatography [10]. The sheep received 0.5, 1 and 2 mg intramusc in complete Freund's adjuvant at two-week intervals followed by a booster injection of 1 mg 2 weeks late HRP, grade IV, RZ = 0.6, was obtained from Miles-Seravac, Maidenhead, Berks., England; glutaraldehyd as a 25% aqueous solution from Schuchardt, 8000 München, W. Germany; lactalbumin from DMV De Meijerij, Veghel, the Netherlands; 5-aminosalicyclic acid from Ega-Chemie, 7924 Steinheim/Albuch, Germany; hydrogen peroxide 30% and microcrystalline cellulose from Merck; Lyphogel (a product of Gelma Instrument Company) from Hawsley and Sons, Lancing, Sussex, England; CNBr from Fluka A.G., Buchs, Switzerland.

2.2. *Equipment*

Ultracentrifuge runs were performed in an International Equipment Company B-60 preparative ultracentrifuge. Absorbance measurements were made with a Gilford 300-N micro-sample spectrophotometer and with an LKB Uvicord.

2.3. *Preparation of immune adsorbents*

Antibodies against HCG and against rabbit γ-globu (RGG) were coupled to cellulose. γ-Globulin fraction of antisera were prepared by precipitation with solid Na_2SO_4 (160 mg/ml for sheep sera, 180 mg/ml for rabbit sera) at room temperature. These γ-globulin fractions were coupled to either *m*-aminobenzyloxymethyl cellulose which had been reprecipitated from a cuprammonium solution and diazotized by Gurvich's method, as described by Campbell and Weliky [11], or to microcrystalline cellulose activated with CNBr [12, 13]. The immune adsorbents are designated as follows

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** USA patent application 762120, filed 24th September 1968; Dutch patent application 7018838, filed 28th December 1970.

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Coupling method	HCG antibodies	RGG antibodies
Diazotation	(a-HCG)-I	(a-RGG)-I
CNBr	(a-HCG)-II	(a-RGG)-II

2.4. *Preparation of HCG–HRP conjugates*

5 mg HCG (5000 IU/mg) and 20 mg HRP were dissolved in 2 ml 0.05 M phosphate–citrate buffer pH 7, containing 1% glutaraldehyde and shaken for 2–3 hr at room temperature. Insolubilized protein was removed by centrifugation at 1000 *g* for 10 min. Excess glutaraldehyde was removed by gel filtration with Sephadex G-50. The enzyme-containing fractions were pooled, concentrated by addition of an appropriate amount of lyphogel, layered over a sucrose density gradient (20–60%), and centrifuged at 5° for 16 hr at 283,000 *g*. Fractions were collected after perforation of the bottom of the tube.

2.5. *Haemagglutination inhibition test for HCG*

The procedure described by Wide [14] was used, with some modifications [9].

2.6. *Peroxidase assay*

A substrate solution was prepared by dissolving 80 mg 5-aminosalicyclic acid in 100 ml 0.02 M phosphate buffer pH 6.0, to which 10 μl 30% H_2O_2 was added. A 1 ml enzyme sample containing 1–20 mIU was added to 1 ml of the freshly prepared substrate solution. The absorbance at 450 nm was read after incubation for 1 hr at room temperature.

2.7. *Enzyme-immunoassay procedures*

(A) *Solid phase (SP) method*: A 0.5 ml sample containing HCG and 0.4 ml (a-HCG)-I or -II suspension were incubated during rotation for 2 hr. 0.1 ml conjugate solution containing about 10 mIU HRP activity was added, and incubation was continued for 2 hr. After centrifugation for 5 min at 2000 rpm, 0.5 ml was taken from the supernatant, diluted 1:1 with 0.02 M phosphate buffer, and assayed for HRP-activity as described under 2.6.

(B) *Double antibody solid phase (DASP) method [15]*: A 0.5 ml sample containing HCG and 0.1 ml diluted a-HCG serum were incubated for 30 min. Then 0.1 ml conjugate solution containing about 10 mIU HRP activity was added and incubation continued for another 30 min. (a-RGG)-I or -II suspended in 0.3 ml was added, and the reaction mixture was shaken for 2 hours. Centrifugation and enzyme-assay were performed as described above.

All solutions were made in 0.02 M phosphate buffer pH 6.0, containing 0.1% lactalbumin. 1 g lactalbumin was stirred with 100 ml phosphate buffer for 15 min, filtered over Hyflo filter aid and diluted to 0.1% on the basis of the measurement of A_{280} using a standard curve. All procedures took place at room temperature.

No HCG was added and the first incubation was omitted for the determination of the percentage enzymatically active conjugate which could be bound to excess a-HCG. This property will be indicated as 'immune reactivity' of the conjugate. The first incubation was also omitted when antiserum dilution curves were determined.

3. Results

Density gradient centrifugation was used to separate HCG-bound HRP from the unreacted material. A typical case is shown in fig. 1. A reasonably sharp

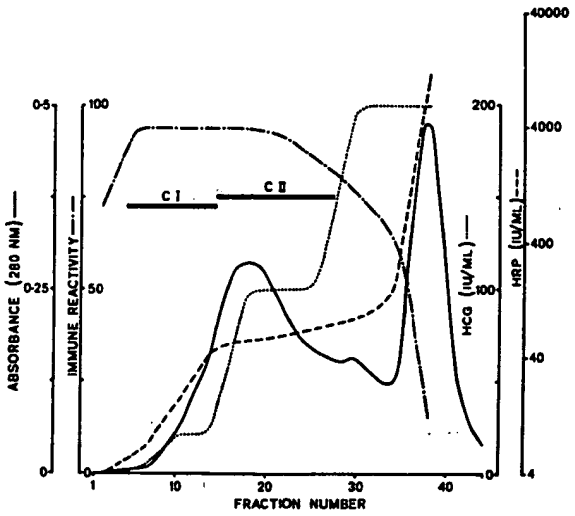

Fig. 1. Fractionation of an HCG-HRP conjugate by density gradient centrifugation (section 2.4). Fractions were collected from the bottom of the tube and pooled as indicated by bars.
—— absorbance at 280 nm
. . . . . HCG in IU/ml (immunoassay)
- - - - - HRP in IU/ml
-.-.-.- immune reactivity (cf. section 2.7)

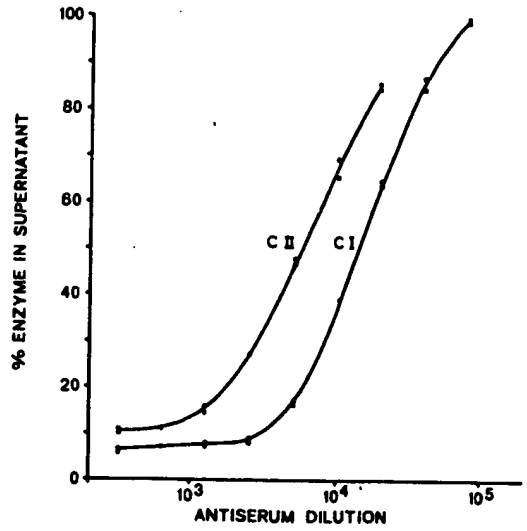

Fig. 2. Antiserum dilution curves of a high- and low- HCG/HRP-ratio conjugate (CII and CI respectively). 100% enzyme activity equals an A_{450}^{1cm} of about 0.400 in the enzyme-assay.

separation between conjugated and unconjugated HRP is obtained, as can be seen from the decrease in the immune reactivity (cf. section 2.7) around fraction 30. Fractions 5–27, showing more than 80% immune reactivity, were chosen for use in our assays. They contained

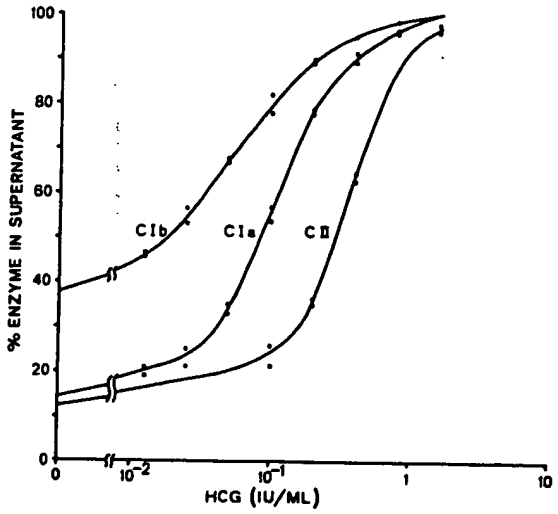

Fig. 3. Standard curves for HCG assay using a low (CI) and a high (CII) HCG/HRP ratio conjugate. The antiserum dilutions for the various curves were: CIa: 1/5000; CIb: 1/10000; CII: 1/1250. 100% enzyme activity equals an A_{450}^{1cm} of about 0.400 in the enzyme-assay.

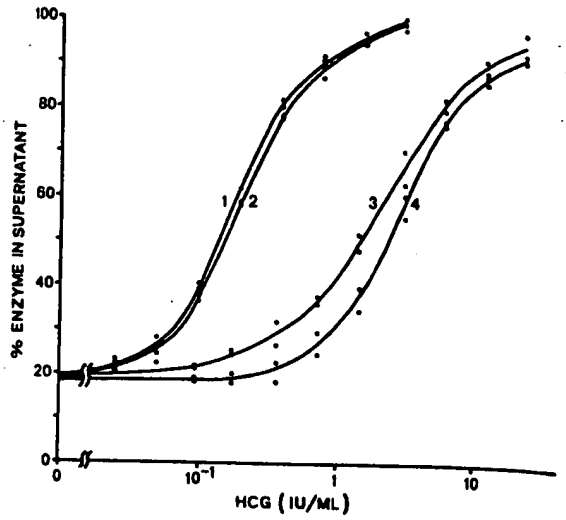

Fig. 4. Comparison of solid phase and double antibody solid phase systems in enzyme-immunoassay, using conjugate CII. 100% enzyme activity equals an A_{450}^{1cm} of about 0.400 in the enzyme-assay.
Curve 1: (a-RGG)-II, } Antiserum dilution
Curve 2: (a-RGG)-I, } 1/2000
Curve 3: (a-HCG)-II, 1.2 mg/tube
Curve 4: (a-HCG)-I, 0.1 mg/tube
Details are described in section 2.

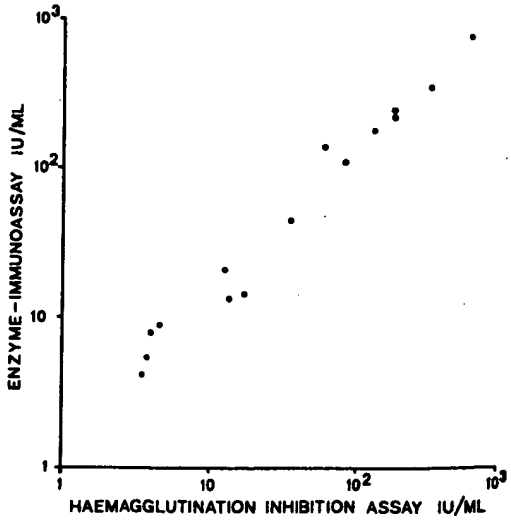

Fig. 5. Correlation between HCG-content of 15 pregnancy urines as measured by haemagglutination inhibition assay and by enzyme-immunoassay.

some 5% of the total enzyme activity. The faster sedimenting conjugates in fractions 5–14 showed a lower HCG/HRP ratio than the conjugates in fractions 15–27. Therefore, they were combined into two pools, CI and CII respectively.

As shown in fig. 2, CII requires 2–3 times more a-HCG than CI to attain the same percentage binding of its enzyme activity. Standard curves obtained with these pools showed a corresponding difference in sensitivity, CI giving the most sensitive assay (fig. 3). As in the radio-immunoassay, the sensitivity can be raised by using a higher antiserum dilution. The results shown in figs. 2 and 3 were obtained with the DASP method (section 2.7B), using (a-RGG)-I (section 2.3).

A comparison of the SP and DASP methods is shown in fig. 4. Little difference in sensitivity was found between the DASP-systems employing (a-RGG)-I and -II. However, both SP systems were markedly less sensitive, (a-HCG)-I giving the least sensitive system. The applicability of the test system was illustrated by the determination of HCG in urines from 15 pregnant and 15 non-pregnant women, both by DASP enzyme-immunoassay – adjusted to a sensitivity level of 0.4 IU/ml – and by a haemagglutination inhibition assay with a sensitivity of 1 IU/ml. The correlation between these test methods is shown in fig. 5. The HCG concentrations in all non-pregnancy urines were below the detection of either test.

4. Discussion

Density gradient centrifugation could be used for obtaining conjugates with an 'immune reactivity' (cf. section 2.7) of 80% or more. Since the immune reactivity of the unpurified conjugate is about 5%, a purification of at least 16-fold was achieved. The low figure for the unpurified conjugate may reflect the limited reactivity of HRP [16].

Ultracentrifugation also separated conjugates with high and low HCG/HRP ratios. Whether this is a separation between HCG/HRP-conjugates with different compositions or between such conjugates and 'pure' polymerized HCG has not yet been investigated. The antibody concentration necessary to obtain e.g. 50% binding of a conjugate depended on the HCG/HRP ratio. Since the attainable sensitivity depends on this antibody concentration, sensitivity requirements determine which conjugate is to be used in a given test system.

Separation between antibody-bound and free conjugate is as important in enzyme-immunoassay as it is in radio-immunoassay. We used the SP and the DASP method [15]. The latter was found to be superior for two reasons:

(a) Higher sensitivity. This might be explained by the assumption that some of the antibodies on the (a-HCG)-immune adsorbents are accessible for HCG, but not for the high-molecular-weight conjugates, while others are accessible for all reaction partners. Excess of (a-HCG)-cellulose impairs the sensitivity of the test system while excess of (a-RGG)-cellulose does not.

(b) Better duplicate determinations, possibly because an (a-HCG) solution can be added more accurately than an (a-HCG)-cellulose suspension. The amount of (a-RGG)-cellulose in DASP systems is not critical, since it has to be used in excess.

The sensitivity of the enzyme-immunoassays is within the range of haemagglutination inhibition or complement fixation assays, when using SP systems. DASP systems were 10–20 times more sensitive, but did not reach the high sensitivity of radio-immunoassay. The enzyme-immunoassay could be performed on urine samples and this may be important for clinical applications.

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